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14. ABSTRACT

The goal of this project is to develop an assay to measure the binding a common food-borne carcinogen (PhIP)to human serum albumin (HSA), an abundant protein in blood that frequently binds to small molecules in the blood stream. Measurement of HSA-bound PhIP may allow determination of the exposure (dosimetry) of large populations of humans to PhIP. Such critical data are almost never included in epidemiology studies owing to low detection sensitivity of current methods. Based upon our earlier results in humans demonstrating that PhIP binds to HSA, we are developing an ultrasensitive mass spectrometry assay that depends upon competitive binding of adducted HSA to an antibody saturated with a radiocarbon labeled PhIP-bound peptide. This assay will allow quantitative measurement with the naturally occurring substrate from archived human samples. We report progress towards antibody development that will enable the assay to be realized.

15. SUBJECT TERMS
protein adduct, PhIP, biomarker, prostate cancer, accelerator mass spectrometry

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Introduction:

Prostate cancer is the second leading cause of cancer death in men in the United States, with African-Americans having the highest rate of prostate cancer in the world. A few epidemiology studies have indicated that exposure to PhIP, a rodent prostate carcinogen formed in meat during cooking, may be an important risk factor for prostate cancer in humans. There is also some evidence that African-American children have a 2-3 fold higher exposure to PhIP than White American children. Children are an important population to study because carcinogen exposure during development may lead to increased prostate cancer risk in later life. However, the epidemiology data are based upon PhIP exposure estimates from dietary questionnaires, rather than measurement of molecular markers that more accurately quantify an individual's internal dose and potential cancer risk. Therefore, a highly sensitive biomarker assay is urgently needed to clarify the role of PhIP in prostate cancer.

The goal of this project is to develop an assay that can be used to more accurately quantify human exposure to PhIP and potential prostate cancer risk. Our hypothesis is that an Accelerator Mass Spectrometry-based method can be developed to measure protein adducts of PhIP in the blood of humans. This will provide a measure of the internal dose, as well as the capacity for carcinogen bioactivation to a form that can initiate the cancer process. In a proof-of-principle study, we have synthesized a model of a PhIP-protein adduct that is expected to form in vivo via reaction with PhIP metabolites and human serum albumin (HSA), the most abundant extra cellular protein in blood.

Our aims are to 1) Characterize the adducts formed from the reaction of the carcinogen PhIP with a peptide derived from the sequence of human serum albumin 2) Develop an ultrasensitive radiommunoassay for PhIP albumin adducts so that they can be assessed in populations of people. 3) Measure PhIP albumin adduct levels in archived blood samples that were obtained from human male patients.

Body:

In the first year of this award PhIP was shown to react predominantly at the cysteine residue of a model peptide containing all twenty amino acids. Additionally, PhIP was shown to generally react with HSA at solvent exposed residues to form both stable and unstable (heat and acid labile) adducts. In the second year of the project conditions were optimized to quantify the labile adducts and trypic digests of HSA from patients containing stable PhIP adducts were characterized by Fourier transform cyclotron resonance mass spectrometry (FTMS). The data support that there are multiple sites of PhIP binding. At the end of the second year Karen Dingly and Ahn Tyet Tran (The PI and posdoc for the project) left the laboratory and Paul Henderson became PI. The aims were refined to focus on assay development using a small peptide whose sequence is derived from solvent exposed portion of HSA. Notably, the sequence is unique compared to rabbit and mouse serum albumins, which will allow antibodies to be raised in either of those animals with the PhIP-adducted peptide. During final year this grant we have worked on Specific Aims 1 and 2 of the proposal. We identified a cysteine adduct that was formed between N-acetoxy PhIP in HSA and a related peptide model. The progress since the PI change is described in greater detail as follows:

Specific aim #1: Characterize the adducts formed from the reaction of the carcinogen PhIP with a peptide derived from the sequence of human serum albumin. **The goal of this aim is to synthesize sufficient quantities of a PhIP-adducted peptide adduct for characterization by mass spectrometry and then prepare sufficient quantities of the compound for antibody production.**

We report characterization of an in-vitro adduct formed by N-acetoxy-PhIP with a FLAG-tagged human serum albumin peptide fragment. The peptide sequence was derived from the known sequence of HAS (He, X.M. and Carter, D.C. Nature 358, 209-215 (1992). The peptide was adducted to PhIP, HPLC purified and characterized by FT-MS as described below and shown in Figure 1:

Materials: The peptide DYKDDDDKAFAQYLQQCPFEDHVK[CtBu][NH2] (CtBu= t.-butylthio-L-cysteine; NH2= carboxy terminus modification) was synthesized and purified by Sigma Genosys (The Woodlands, TX). N-acetoxy-PhIP was synthesized and purified as previously described (Brown *et al.*, 2001). HPLC grade solvents and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Covalent Adduct Formation: Adducts were formed as previously described with the some modifications (Chepanoske *et al.*, 2004). Two hundred micrograms of peptide was reacted with an equimolar amount of N-acetoxy-PhIP in 50 mM potassium phosphate at pH 9. Total reaction volume was 1 ml. N-acetoxy-PhIP was dissolved in methanol right before use and added in three aliquots every 20 minutes; the solution was stored at -20°C when not in use. The reaction occurred inside 1.5 ml microcentrifuge tubes on a Thermomixer 5436 (Eppendorf, Hamburg, Germany) at 700 RPM for 60 minutes. Control reactions of only peptide and only N-acetoxy-PhIP were performed simultaneously. After reaction, the products were vacuum-dried. For HPLC purification, samples were taken up in water at a concentration of 1 mg/ml. For FT-MS characterization, samples were vacuum-dried, desalted with $ZipTip_{C18}$ tips (Millipore; Bedford, MA) using a protocol recommended by the manufacturer (Millipore Technical Note TN224, 2000), vacuum-dried again, and then taken up in water at a concentration of 1 mg/ml.

HPLC Methods: An HPLC equipped with an SPD-10AVP UV-Vis detector (Shimadzu Scientific, Inc., Columbia, MD) was used for purification and UV detection. Ten microliters of 1 mg/ml of peptide adduct reaction mixture was loaded onto a 4.6 mm x 250 mm, 5 μm particle size, 120 A pore size, ODS Hypersil column (Thermo Electron Corp, San Jose, CA) with 10% Acetonitrile and 0.1% Trifluoroacetic acid (Solvent A) and 90% Acetonitrile and 0.1% Trifluoroacetic acid (Solvent B) at 1 ml/min at wavelengths of 214 nm and 354 nm. Purifications were done at 100% A for two minutes, increased to 67.5% B for 18 minutes, isocratic at 67.5% B for five minutes, and then decreased to 0% B for 5 minutes.

Fourier Transform Mass Spectrometry was used to measure the adduct formation in conjunction with HPLC purification with UV-Vis detection as shown in Figures 1-4. Figure 2 shows the HPLC trace of the peptide after exposure to mock adduction conditions (no PhIP). The trace is recorded from detection at two wavelength; 214 nm for detection of the peptide and 354 nm for detection of PhIP. The pure peptide eluted from the column at ~17 min with some buffer or

column generated impurities eluting at later times.

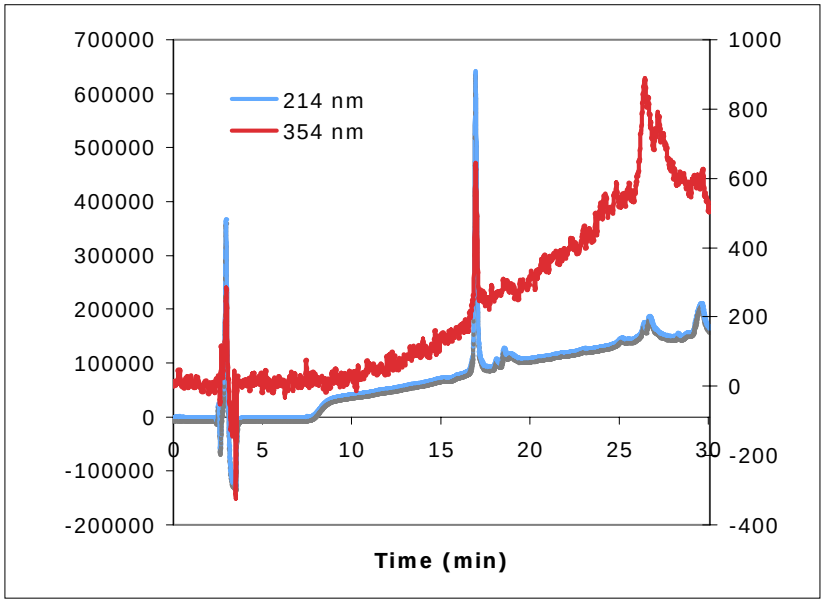

Figure 1: Mock reaction of the peptide DYKDDDDKAFAQYLQQCPFEDHVK reacted in 50 mM potassium phosphate, pH 9 for 60 minutes at 37°C without N-Acetoxy-PhIP. The major peak observed at 16.9625 minutes is the unmodified peptide.

The mock reaction demonstrated that the peptide is stable to the reaction conditions and showed possible contaminants in the buffer system. Figure 2 shows the reaction of N-acetoxy-PhIP with the peptide. Differences from the mock reaction include peaks at ~11 min and several new peaks between 17 and 20 minutes. The peak at 11 min corresponds to unreacted N-acetoxy-PhIP. The remainder of the new peaks showed absorbance at both 214 nm and 354 nm, indicating possible adduction of the PhIP to the peptide. Importantly, the new peaks did not coelute with any significant impurities. The yield of the reaction was approximately 2-3 percent based on the area under the curve of the absorbance detected at 214 nm. As shown in Figure 3., the crude PhIP-adducted peptide sample was injected into the LC-FTMS and high-resolution mass spectrum of the eluting peptides was obtained. The resulting plot shows on the x-axis the mass-to-charge ration for the triply charged peptide ion and on the Y-axis the elution time from the HPLC column (note that the elution times are ~8-9 minutes later than the previous chromatograms due to use of a different HPLC instrument and a buffer pH of 4.0 in order to accommodate the positive ion mode of the mass spectrometer). The band on the left side of the figure represents the unadducted peptide and the band on the right indicates the peptide with one PhIP adduct. Importantly, the mass spectrometer shows only one species corresponding to the PhIP adducted peptide, whereas the UV-Vis detector indicates that at least four species are present. We interpret this as four different amino acids on the peptide acting as substrates for PhIP adduction. Even though there was a slight molar excess of PhIP in the reaction, there was a large molar excess of functional groups on the peptide, which apparently results in at most one PhIP adduct per peptide.

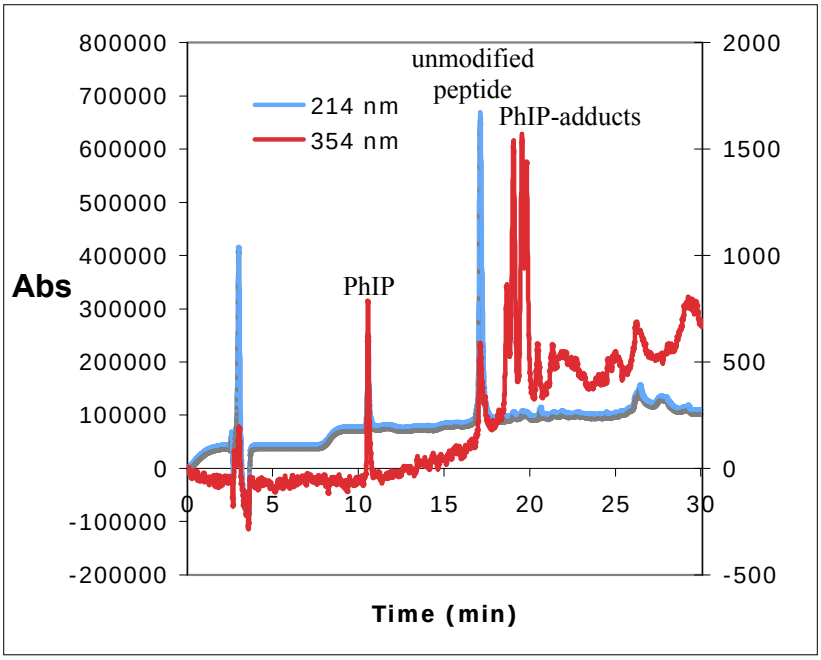

Figure 2: Chromatogram of 10 ug of the peptide DYKDDDDKAFAQYLQQCPFEDHVK reacted in 50 mM potassium phosphate, pH 9 for 60 minutes at 37°C with N-Acetoxy-PhIP. The major peak for unadducted peptide was observed at 16.9625 minutes. The peaks with and absorbance at 354 nm between 16 and 20 minutes are believed to be adducts.

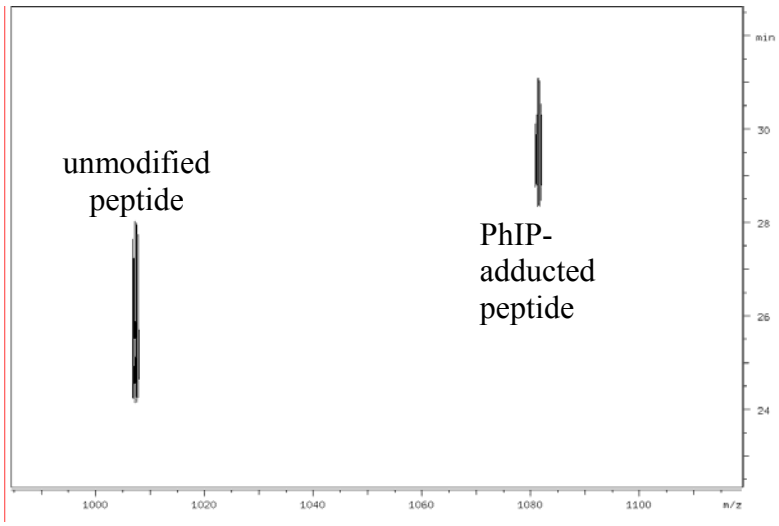

Figure 3: Two-dimensional LC-FTMS plot of native and PhIP-adducted peptides. The peptide on the left shows and average mass of 3,107.708 Da. (The theoretical mass is 3,108.47). The spot on the left eluted at 24-28 minutes with 1006.841 m/z (+3 charged ion). The spot on the right

eluted at 28-32 minutes with a 1080.877 m/z and represents the major peptide-PhIP adduct.

Discussion:

More analysis needs to be applied to these adducts. Tandem mass spectrometry will be performed on putative adducts in order to identify the residue to which the PhIP is binding. We need to verify that the adduct is formed at the internal and not the terminal cysteine residue, which is needed for conjugation to the KLH protein required for antibody production. If the PhIP adducts at the terminal cysteine, our protection strategy needs to be changed; protection of this residue is critical for the binding of keyhole limpet hematocyanin for antibody development.

HPLC elution parameters need to be optimized for better adduct resolution. Because the adducts currently elute too closely together, it is difficult to get optimal sensitivity, accuracy, and precision on the FTMS. The first strategy that will be applied will be elongating the gradient time with reverse-phased HPLC; this will result in better peak resolution on the chromatogram and more efficient fraction collection. The second strategy that will be used is to apply two-dimensional HPLC to the chromatography. In addition to reversed-phase HPLC of the crude reaction mixture, the adducts will be further resolved with anion exchange HPLC. The second type of chromatography will enhance the separation of the adducts so that they may be analyzed with more precision on the FTMS.

Discussion:

The results presented above indicate that the HSA-derived peptide is a reasonable candidate for an antibody assay, since we were able to detect a predominant major adduct as well as several products of lower abundance. These products all had the same mass consistent with the binding of PhIP to a single site. Future effort will focus on production conjugation of the major adducted peptide product to KLH and subsequent generation of antibodies needed for the assay.

Key Research Accomplishments:

During the third year of this grant, we have shown that:

- Single PhIP-peptide adducts form on a fragment of HSA.
- At least 3-4 adducts form, which indicates that multiple amino acids may be involved in PhIP adduction to HSA.
- In order for an antibody assay to quantify PhIP-HAS adduction, multiple antibodies are likely to be needed, which can be generated simultaneously in one animal as polyclonals.

Reportable Outcomes:

Cindy Lou Chepanoske, Karen Brown, Kenneth W. Turteltaub, and Karen H. Dingley. Characterization of a peptide adduct formed by *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a reactive intermediate of the food carcinogen PhIP. *Food and Chemical Toxicology* 42 (2004) 1367-1372.

Ahn, S., Ubick, E. and Dingley, K. Characterization of the protein adducts formed by the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in humans. Genetic and Environmental Toxicology Association of Northern California, 7th May, 2004, Oakland, CA. **Sylvia won first prize for this poster in the poster competition**.

Ahn, S., Ubick, E. and Dingley, K. Characterization of the protein adducts formed by the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in humans. 6th Annual UC Davis Conference for Environmental Health Scientists, August 30, 2004, The Embassy Suites, Napa, California.

Dingley, K.H. Ultra-sensitive quantitation of Heterocyclic amine adducts using accelerator mass spectrometry: from risk identification to chemoprevention'. United Kingdom Environmental Mutagen Society, 27th Annual Meeting, University of Loughborough, July 4-7, 2004.

Dingley, K.H. 'Quantitation of macromolecular targets of heterocyclic amine carcinogens at low dose'. 228th American Chemical Society National Meeting, Philadelphia, PA, August 22-26, 2004.

Dingley, K.H. 'Ultra-sensitive quantitation of heterocyclic amine adducts using accelerator mass spectrometry: from risk identification to chemoprevention'. Tenth Annual Cancer Research Symposium, UC Davis Cancer Center, October 20-21, 2004.

Tran, A.-T. T., Young, N.L., Ahn, S.J., Dingley, K.H. Structural Characterization of DNA-MeIQx adducts. 10th Annual Cancer Research Symposium, 10/20/04 – 10/21/04, UC Davis Cancer Center, Sacramento, California.

Tran, A.-T. T., Young, N.L., Palmblad, M., Ahn, S.J., Dingley, K.H. Structural Determination of the Adducts formed by Heterocyclic Amines with Biomacromolecules. 6th Annual UC Davis Conference for Environmental Health Scientists, 8/30/04, The Embassy Suites, Napa, California.

Tran, A.-T. T., Young, N.L., Ahn, S.J., Butler, B.K., Lebrilla, C.B., Benner, W.H., Cosman, M., Maxwell, R.S., Dingley, K.H. Structural Determination of the Adducts formed by Heterocyclic Amines with Biomacromolecules. CMS Postdoctoral Symposium 2004, 7/27/04, Lawrence Livermore National Laboratory, Livermore, California.

Employment/Research Opportunities

Over the period of this grant, we have supported a post-doctoral fellow (Anh-Tuyet Tran) and two biomedical scientist (Sylvia Ahn and Kristen Stivers), to work on this project. This was the first opportunity for Ahn, Sylvia and Kristen to work in prostate cancer research.

Conclusions:

The observed formation of PhIP-cysteine adducts justifies the continued development of an assay that can quantitate these adduct levels in humans. The ultimate question of whether or

not these adducts can serve as markers of prostate cancer susceptibility, initiation or progression can only be answered with such an assay. Over the next months we will focus efforts on synthesizing sufficient quantities of peptide with the major PhIP-cysteiene adduct as a substrate for raising monoclonal antibodies in mice, and the use of those proteins to develop an AMS-based assay that is sensitive enough to detect the adducts in archived samples from humans dosed with low amounts of 14C-labeled PhIP.

So What? Quantitation of exposure and individual variation in response to exposure is critical to understanding the influence of environment and genetic predisposition on prostate cancer. Currently, there does not exist an adequate method to measure exposure and binding of carcinogens to biological molecules in humans at relevant level such as at the concentrations of PhIP known to be present in cooked meats. Our PhIP-HSA assay will be applicable to human samples and may provide a measurement of carcinogen-protein adducts that can be correlated to exposure and genetic susceptibility.

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